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<b>13. ABSTRACT (Maximum 200 Words)</b> Our objective is to use X-ray crystallography to determine the basis for specific interactions between the Androgen receptor and its DNA targets, in order to understand how the receptor can recognize two different bipartite DNA response elements with diametrically opposing arrangements. Our research has now identified a variant of the AR DNA binding domain that yields large, single lattice crystals when bound to a direct repeat response element. The 3-dimensional crystal structure of the androgen receptor (AR) DNA binding domain (DBD) bound to a selective ADR3, determined at 3.1 A resolution, reveals an unexpected head-to-head arrangement of the two promoters, rather than the expected head-to-tail arrangement seen in nuclear receptors bound to response elements of similar geometry. Compared to the glucocorticoid receptor (GR), the DBD dimmer interface of the AR has additional interactions that stabilize the AR dimmer and increase the affinity for non-consensus response elements. This increased interfacial stability compared to the other steroid receptors may account for the selective binding of AR to ADR3 response elements.				
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## Introduction

The androgen receptor (AR) is a member of the steroid and nuclear receptor superfamily and it plays a central role in the development and progression of prostate cancers. The class I steroid receptors (androgen (AR), glucocorticoid (GR), mineralocorticoid (MR), progesterone (PR)) have identical palindromic consensus response elements and are nearly identical in their core DNA binding domains. Recent work has uncovered a second androgen response element that retains the canonical half site sequence, but has half sites that are arranged as a direct repeat (ADR-3) instead of a palindrome. Our objective is to determine the stereochemical basis for specific interactions between the Androgen receptor and DNA targets, to understand how the receptor can recognize two different bipartite DNA response elements with diametrically opposing arrangements, and to understand the stereochemical role that the C-terminal extension and hinge regions of the AR DNA binding domain (dbd) play in the recognition of specific DNA sequences. We are using X-ray crystallography to visualize complexes between the androgen receptor and specific DNA targets. Once the structures are solved, the protein-protein and protein-DNA contacts that lead to specific binding will be analyzed. These structures could be used to design novel classes of anti-androgens that disrupt the protein-protein or protein-DNA interactions of the androgen receptor. These compounds may be extraordinarily potent inhibitors of AR activation, since they would fundamentally de-couple the receptor from its interactions with target genes.

## Body

Task 1: To solve the structure of the AR dbd bound to a direct-repeat type response element. During the first project period, we succeeded in optimizing our crystals of the AR dbd bound to direct repeat DNA targets and were able to grow reproducibly large, single lattice crystals of the AR dbd-ADR3 complex. During the latest project period, we have solved the structure of this complex, using a combined MAD and molecular replacement approach. Briefly, crystals were gradually equilibrated into reservoir solution supplemented with 35% glycerol before being flash cooled in liquid nitrogen. Diffraction data were collected at  $-180^{\circ}\text{C}$  on beamline 22ID at the Advanced Photon Source using a MarResearch CCD detector. Data were indexed and reduced using HKL2000. Initial sites for the zincs were found using SOLVE and data from the peak anomalous wavelength. Four zinc sites were located. Experimental phases were generated using these sites and in the anomalous difference Fourier maps, the four zinc sites had peaks of greater than  $30\sigma$ , while the next highest peak was  $3\sigma$ , indicating there was one AR dimer in the asymmetric unit. Visual inspection of the zinc sites revealed that the proteins were arranged in a palindromic orientation. This led to construction of a molecular replacement model using the ER DBD-IR3 structure (PDB code 1HCQ). Due to its higher sequence homology to AR, the ER DBD was replaced with the core GR DBD (PDB code 1GLU) using least squares fitting. A molecular replacement solution was obtained using MOLREP. MAD phases were calculated using the remote and peak wavelength data to  $3.4\text{\AA}$  and used in all rounds of refinement. Refinement of the structure was done using CNS and the MLHL target at  $3.1\text{\AA}$  resolution. Model building was done using O. Even at  $3.1\text{\AA}$  resolution, the number of reflections used was 8-fold greater than the number of modeled atoms due to the very large (>80%) solvent content of the crystal, allowing for restrained individual B-factor refinement in later rounds. An overview of the structure is presented in Figure 1.

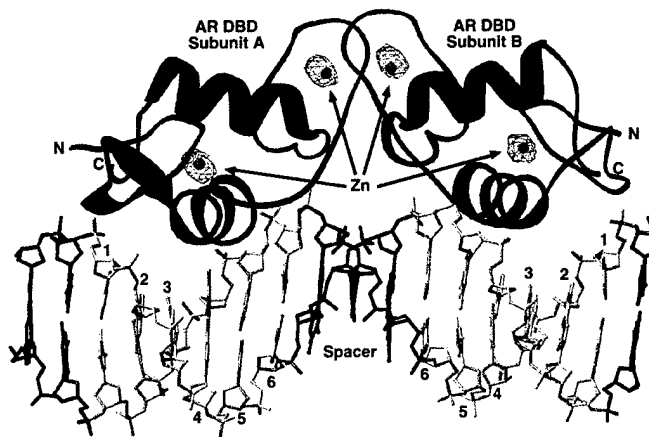
Task 2: To determine the structure of the AR dbd bound to a palindromic response element. The discovery that the AR dbd C552A mutant yields well-diffracting crystals when bound to direct repeat response elements is an important milestone that has been exploited in the crystallization experiments using palindromic response elements. To date, small crystals of the AR dbd C552A in complex with an IR3 palindromic response element have been obtained, but these are not of suitable size for diffraction analysis yet. In a parallel effort, we have also begun an NMR analysis of the interaction of the AR dbd with both ADR3 and IR3 response elements.

The goal of these highly focused experiments is to use NOE analysis to observe the interaction between the methyl groups of Val465 of the AR dbd and the C5 methyl group of the T at position 4 of the AR response element. If the AR dbd is bound to an IR3 response element in an inverted repeat orientation, we expect to observe 2 distinct NOEs – one from each protein-DNA half-complex. On the other hand, if the AR dbd is bound as an inverted repeat (as seen in the crystal structure described above) to a direct repeat response element such as the ADR3, then we would expect to see only one NOE between the Val465 and T, corresponding to the specifically-bound complex. This should address both the question of how the AR dbd is bound to traditional inverted repeat response elements, as well as provide important confirmation of the results of the AR dbd – ADR3 crystallography experiments.

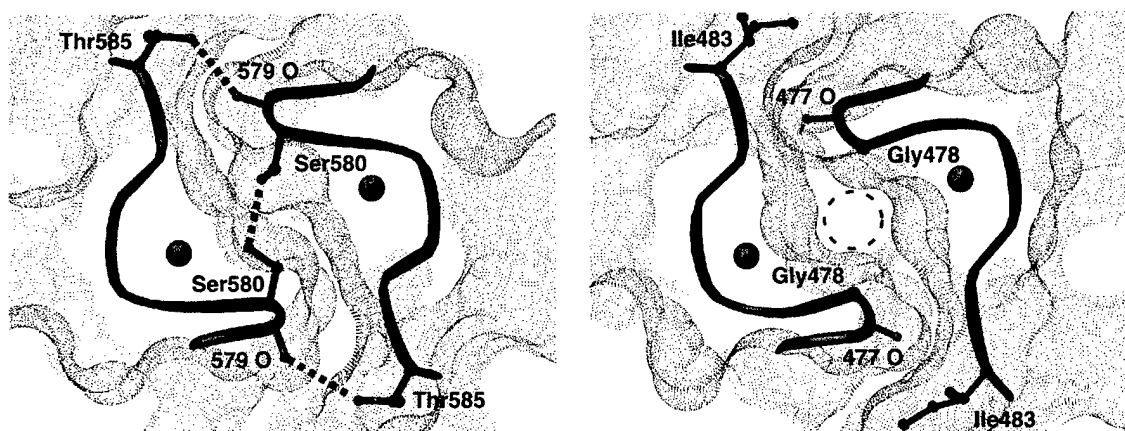
## A

## AR DBD on DR3 DNA

**Figure 1.** Overview of the structure of the AR dbd bound to ADR3 DNA. The two AR dbd subunits are shown in red and blue. The two DNA 1/2 sites are in gold, and the flanking and spacer sequences are in black. The model is superimposed on an electron density map calculated from MAD phases collected at the zinc edge, which, since they coincide with the Zn atoms, confirms the orientation of the two dbd subunits.



**Task 3:** Analysis and comparison of structures. As mentioned in the previous years report, the analysis of the VDR dbd-DR-3 complex has been completed and the work has been published (Shaffer, P.L. & Gewirth, D.T. (2002) "Structural Basis of VDR-DNA Interactions on Direct Repeat Response Elements." *EMBO J* 21, 2242-2252.). With the structure of the AR dbd-ADR3 complex solved we have been able to make an informed comparison with both the VDR complex as well as the previously-determined structure of the glucocorticoid receptor bound to an inverted repeat response element. In summary, the structure of the AR dbd-ADR3 complex exhibits a more extensive and stronger dbd dimer interface than the corresponding interface in the GR dbd-DNA complex (Figure 2), and this stronger interface accounts for the ability of AR to bind to AR selective response elements.



**Figure 2.** Comparison of key differences in the AR dbd (left) and GR dbd (right) dimer interfaces. The AR dbd dimer interface has hydrogen bond interactions between Thr585 and the carbonyl oxygen of G579, and reciprocal hydrogen bonds between Ser580. The GR dbd, on the other hand, exposes the side chain of I483 to solvent, and exhibits a "glycine hole" which makes no productive interactions between subunits. Zinc atoms are shown as grey spheres, and the two dbd subunits of the dbd dimer are shown in red and blue for both AR and GR.

### Key Research Accomplishments

- Discovery of an AR dbd mutational variant (C552A) that maintains DNA binding capability and results in single lattice crystals.
- Discovery of well-diffracting crystals of the AR dbd C552A-ADR3 complex.
- Determination of the structure of the AR dbd-ADR3 complex.

### Reportable Outcomes

1. A manuscript describing the structure of the AR dbd-ADR3 complex has been accepted for publication in PNAS and is now in press (Shaffer, Jivan, Dollins, Claessens, & Gewirth, "Structural Basis of Androgen Receptor Binding to Selective Androgen Response Elements").
2. A presentation of these results was given on 14 November, 2003 at the University of Leuven, Belgium, and on February 5, 2004 at Bowling Green State University, Bowling Green, Ohio.
3. The atomic coordinates of the AR dbd-ADR3 complex have been deposited with the Protein Data Bank (PDB), accession code 1R4I.

### Conclusions

We have determined the structure of the AR DBD bound to an idealized steroid DR3 response element. Based on earlier studies of the Vitamin D receptor DBD (Shaffer and Gewirth, 2002), which also binds to a DR3-type response element, we expected the tandem arrangement of half-sites to direct head-to-tail binding of the AR DBD to the DNA. Surprisingly, however, the AR DBDs bind to the direct repeat response element as head-to-head symmetrical dimers. As a result of this mismatch between receptor dimer and response element arrangement, one AR DBD is bound to a high affinity cognate half-site, while the partner DBD is bound to a lower affinity half-site. This indicates that the energetic penalty incurred by binding to a less favored half-site sequence is more than offset by maintaining the preferred IR3-type dimer interface. This is analogous to an earlier observation that the GR DBD maintains the IR3 dimer interface and spacing even when challenged with an IR4 response element (Luisi et al., 1991).

Both the AR and the GR exhibit similar interactions with steroid response elements, yet the AR exhibits consistently stronger binding to direct repeat type response elements than does the GR. Some of this difference in affinity may be attributable to differences in the CTE of each DBD, although in the case of both GR and AR these regions were disordered in the crystal structure and may contribute only to general electrostatic interactions without affecting selectivity or discrimination. Within the core of the DBD, however, the protein-DNA interactions are nearly identical for both receptor DBDs, and much of the difference in response element affinity is therefore likely to reside in the ability of each receptor to cooperatively form head-to-head dimers on bipartite response elements where the interaction with one or both hexameric half-sites is non-optimal.

The second zinc module has previously been shown to be necessary for AR to bind cooperatively to ADR3s (Schoenmakers et al., 1999). The steroid receptor DBD dimerization interface is contained within this module, and between AR and GR it differs at just four positions. The increased AR dimer affinity can be explained by two of these four substitutions: one in the D-box, and the other two residues beyond. In the D-box, AR is the only steroid receptor that has a

Ser residue at the second position -- Ser580 -- and this serine packs into the core of the dimer interface, making both van der Waals interactions and a cross-subunit hydrogen bond. All other steroid receptors have a Gly at this position that leaves a void in the interface and lacks this additional hydrogen bond. Two residues beyond the D-box, an Ile to Thr substitution in AR allows both a favorable cross-subunit sidechain-to-backbone hydrogen bond, and removes the non-polar Ile side chain from exposure to solvent. Together these two substitutions appear to account for the stronger AR dimer interface. This in turn allows the receptor to bind to a more diverse set of response elements with higher affinity and cooperativity, compared to the GR.

Biochemical evidence for the increased cooperativity of the AR DBD dimer correlates with these structural observations. All the steroid receptors (MR, PR, GR, and AR) show a 5- to 10-fold lower affinity for the naturally occurring PB-ARE-2 DR3-type element compared to the C3(1) IR3-type element (Schoenmakers et al., 2000). Importantly however, the AR DBD binds 3- to 10-fold better to both elements relative to the other steroid receptors. Thus, the binding constant for AR on an apparent DR3 target ( $23 \pm 5$  nM) is the same as that of the other receptors for the more optimal IR3 element (average of other three is  $23 \pm 9$  nM) (Schoenmakers et al., 1999). Since the concentration of individual steroid receptors in the cell is approximately nanomolar, differences in binding constants of this order are likely to be significant. AR substitutions in the GR dimerization interface show higher affinity binding to both DR3 and IR3 response elements, thus mimicking the behavior of the AR. Together with the structural data, these observations suggest a model where, due to the increased strength of the AR dimer interface, AR-selective gene activation arises from the ability of the AR to bind to IR3 response elements that have a greater deviation from the consensus half-site sequence.

The structure of the AR DBD bound as an inverted repeat to a direct repeat response element highlights the fact that DNA target recognition by hormone receptors is strongly governed by the dimerization behavior of the two interacting protomers, even at the expense of losing specific interactions with the target DNA. To date no physiologically relevant dimerization interface within the steroid receptor DBDs other than the primary one has been observed in structural studies, and attempts to capture such potential alternative interfaces, as described in this report, and previously for GR (Luisi et al., 1991), have been unfruitful. This implies that selective hormone response elements that appear to have alternative arrangements of their hexameric half sites, such as the pemARE with a proposed 5 bp spacer between half-sites (Geserick et al., 2003), may instead simply be further examples of the ability of these receptors to exploit the strength of their DBD dimerization interfaces to accommodate sub-optimal protein-half site interactions. This is likely to be not only a mechanism of response element discrimination, but also an effective way of modulating transcription from different hormone responsive genes.

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